Highlighted Version Sh wing Amendments to Table 3

APPENDIX B

TABLE 3

Crystal Formation by the Hybrid δ-Endotoxins

Strain	NRRL[a] Accession Number	NRRL Deposit Date	Plasmid	Parent δ-Endotoxins	Crystal Formation
EG11060			pEG1065	Cry1Ac + Cry1F	+
EG11062			pEG1067	Cry1Ac + Cry1F	+
EG11063	B-21579	May 24, 1996	pEG1068	Cry1Ac + Cry1F	+
EG11065			pEG1070	Cry1Ac + Cry1F	–
EG11067			pEG1072	Cry1Ac + Cry1F	–
EG11071			pEG1074	Cry1Ac + Cry1F	+
EG11073			pEG1076	Cry1Ac + Cry1F	+
EG11074	B-21580	May 24, 1996	pEG1077	Cry1Ac + Cry1F	+
EG11087			pEG1088	Cry1Ac + Cry1C	–
EG11088			pEG1089	Cry1F + Cry1Ac	–
EG11090			pEG1091	Cry1C + Cry1Ac	–
EG11091			pEG1092	Cry1Ac + Cry1C	+
EG11092	B-21635	Oct. 21, 1996	pEG1093	Cry1Ab + Cry1Ac + Cry1F	+
EG11735	B-21581	May 24,1996	pEG365	Cry1Ab + Cry1F + Cry1Ac	+
EG11751	B-21636	Oct. 21, 1996	pEG378	Cry1Ac + Cry1F	+

[a]The subject cultures have been deposited under conditions that assure that access to the cultures will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 C.F.R. §1.14 and 35 U.S.C. §122. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action. The subject culture deposits will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, *i.e.*, they will be stored with all the care necessary to keep them viable and uncontaminated for a period of at least five years after the most recent request for the finishing of a sample of the deposit, and in any case, for a period of at least 30 (thirty) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the cultures. The depositor acknowledges the duty to replace the deposits should the depository be unable to furnish a sample when requested, due to the condition of the deposits.

All restrictions on the availability to the public of the subject culture deposits will be irrevocably removed upon the granting of a patent disclosing them.

Cultures shown in Table 3 were deposited in the permanent collection of the Agricultural Research Service Culture Collection, Northern Regional Research Laboratory (NRRL), located at 1815 N. University Street, Peoria, IL 61604, under the terms of the Budapest Treaty.--

The δ-endotoxin production for some of the *B. thuringiensis* strains specified in TABLE 3 was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Baum *et al.*, 1990. Equal volume cultures of each *B. thuringiensis* strain were grown in C-2 medium until fully sporulated and lysed. The cultures were centrifuged and the spore/crystal pellet was washed twice with equal volumes of distilled deionized water. The final pellet was suspended in half the culture volume of 0.005% Triton X-100®. An equal volume of each washed culture was analyzed by SDS-PAGE as shown in FIG. 2.

The majority of hybrids involving Cry1Ac and Cry1F formed stable crystals in *B. thuringiensis* A notable exception is EG11088 in which the active toxin fragment would be the reciprocal exchange of EG11063. Two of the three hybrids involving Cry1Ac and Cry1C, EG11087 and EG11090, failed to produce crystal in *B. thuringiensis* even though these reciprocal hybrids mimic the activated toxin fragments of crystal-forming EG11063 and EG11074.

Every strain that was examined by SDS-PAGE produced some level of δ-endotoxin. As expected, however, those cultures identified as crystal negative produced very little protein (*e.g.*, lane e: EG11065, lane f: EG11067, lane j: EG11088, and lane k: EG11090). For reference, typical yields from a crystal forming δ-endotoxin is shown for Cry1Ac (lane a). Several hybrid δ-endotoxins produce comparable levels of protein including EG11060 (lane b), EG11062 (lane c), EG11063 (lane d; SEQ ID NO:10), and EG11074 (lane i; SEQ ID NO:12). The data clearly show that efficient hybrid δ-endotoxin production in *B. thuringiensis* is unpredictable and varies depending on the parent δ-endotoxins used to construct the hybrid.

6.3 Example 3 -- Proteolytic Processing of the Hybrid δ-Endotoxins

Proteolytic degradation of the protoxin form of the δ-endotoxin to a stable active toxin occurs once δ-endotoxin crystals are solubilized in the larval midgut. One measure of the

potential activity of δ-endotoxins is the stability of the active δ-endotoxin in a proteolytic environment. To test the proteolytic sensitivity of the hybrid δ-endotoxins, solubilized toxin was subjected to trypsin digestion. The δ-endotoxins were purified from sporulated *B. thuringiensis* cultures and quantified as described by Chambers *et al.*, 1991. Exactly 250 μg of each hybrid δ-endotoxin crystal was solubilized in 30 mM $NaHCO_3$, 10 mM DTT (total volume 0.5 ml). Trypsin was added to the solubilized toxin at a 1:10 ratio. At appropriate time points 50 μl aliquots were removed to 50 μl Laemmli buffer, heated to 100°C for 3 min., and frozen in a dry-ice ethanol bath for subsequent analysis. The trypsin digests of the solubilized toxins were analyzed by SDS-PAGE and the amount of active δ-endotoxin at each time point was quantified by densitometry. A graphic representation of the results from these studies are shown in FIG. 3.

The wild-type Cry1Ac is rapidly processed to the active δ-endotoxin fragment that is stable for the duration of the study. The hybrid δ-endotoxins from EG11063 and EG11074 are also processed to active δ-endotoxin fragments which are stable for the duration of the study. The processing of the EG11063 δ-endotoxin occurs at a slower rate and a higher percentage of this active δ-endotoxin fragment remains at each time point. Although the hybrid δ-endotoxins from EG11060 and EG11062 are process to active δ-endotoxin fragments, these fragments are more susceptible to further cleavage and degrade at various rates during the course of the study. The 5′ exchange points between *Cry1Ac* and *Cry1F* for the EG11062 and EG11063 δ-endotoxins result in toxins that differ by only 21 amino acid residues (see FIG. 1). However, the importance of maintaining *Cry1Ac* sequences at these positions is evident by the more rapid degradation of the EG11062 δ-endotoxin. These data demonstrate that different hybrid δ-endotoxins constructed using the same parental δ-endotoxins can vary significantly in biochemical characteristics such as proteotytic stability.

6.4 Example 4 -- Bioactivity of the Hybrid δ-Endotoxins

B. thuringiensis cultures expressing the desired δ-endotoxin were grown until fully sporulated and lysed and washed as described in Example 2. The δ-endotoxin levels for each culture were quantified by SDS-PAGE as described by Baum *et al.*, 1990. In the case of bioassay screens, a single appropriate concentration of each washed δ-endotoxin culture was

topically applied to 32 wells containing 1.0 ml artificial diet per well (surface area of 175 mm^2). A single neonate larvae was placed in each of the treated wells and the tray covered by a clear perforated mylar sheet. Larvae mortality was scored after 7 days of feeding and percent mortality expressed as the ratio of the number of dead larvae to the total number of larvae treated (32).

In the case of LC_{50} determinations (δ-endotoxin concentration giving 50% mortality), δ-endotoxins were purified from the *B. thuringiensis* cultures and quantified as described by Chambers *et al.*, 1991. Eight concentrations of the δ-endotoxins were prepared by serial dilution in 0.005% Triton X-100® and each concentration was topically applied to wells containing 1.0 ml of artificial diet. Larvae mortality was scored after 7 days of feeding (32 larvae for each δ-endotoxin concentration). In all cases the diluent served as the control..

A comparison of the Cry1A/Cry1F hybrid toxins by bioassay screens is shown in TABLE 4. The hybrid δ-endotoxins from strains EG11063 and EG11074 maintain the activities of the parental Cry1Ac and Cry1F δ-endotoxins. Furthermore, the hybrid δ-endotoxin from EG11735 maintains the activity of its parental Cry1Ab and Cry1F δ-endotoxins. The δ-endotoxins produce by strains EG11061, EG11062, EG11071, and EG11073 have no insecticidal activity on the insect larvae tested despite 1) being comprised of at least one parental δ-endotoxin that is active against the indicated larvae and 2) forming stable, well-defined crystals in *B. thuringiensis*. These results demonstrate the unpredictable nature of hybrid toxin constructions.

For the data in TABLE 4. All strains were tested as washed sporulated cultures. For each insect tested, equivalent amounts of δ-endotoxins were used and insecticidal activity was based on the strain showing the highest percent mortality (++++).